# A Plant Metabolomic Approach to Identify the Difference of the Seeds and Flowers Extracts of *Carthamus tinctorius* L.

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Abstract: Carthamus tinctorius L. (known as safflower) is a valuable oil plant whose importance is increasing rapidly in the world due to its high adaptation to arid regions. The seeds of this unique plant are especially used in edible oil, soap, paint, varnish and lacquer production. Its flowers are used in vegetable dye production and medicinal purposes beside its features as a coloring and flavoring in food. After the oil is removed, the remaining pulp and plant parts are used as animal feed, and dry straw residues are used as fuel. Beside all these features, its usage as a herbal medicinal plants for various diseases has gained importance on recent years. In this study, it was designed a plant metabolomic approach which transfers all the recent data processing strategies of untargeted metabolomics in clinical applications to the present study. Q-TOF LC/MS-based analysis of the extracts (70% ethanol, hexane, and chloroform) for both seed and flowers was performed using a C18 column (Agilent Zorbax 1.8  $\mu$ M,  $100 \times 2.1$  mm). Differences were observed in seed and fruit extracts and these differences were visualized using principal component analysis (PCA) plots. The total number and intersections of the peaks in the extracts were visualized using peak count comparison graph. Based on the experimental results, the number of the detected peaks for seeds was higher than the ones for the flowers for all solvent systems to extract the samples.

Keywords: Carthamus tinctorius L., extract, metabolomics, metabolite profiling, mass spectrometry.

#### Introduction

Compared to genomics and proteomics, metabolomics is a relatively new field, and many attempts have been made, and are still developing, to effectively outline the metabolomic profiles of living organisms.<sup>1,2</sup>

The conversion of high performance liquid chromatography HPLC devices to ultra performance liquid chromatography (UPLC) devices is an improvement of the last decade.<sup>3</sup> In parallel with the changes in device technology, changes in computer technology have been eye-catching in the last few years. Therefore, analyzing complex matrices via high performance liquid chromatography combined with mass

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spectrometry (LC-MS) and processing the data on recent computational technologies allowed researchers to apply metabolomics approaches to various scientific areas like food technology, microbiological studies, environmental applications, and drug discovery<sup>4-8</sup> beside clinical applications.<sup>9,10</sup>

A new area that makes metabolomics functional is phytomedicine. The research interests using metabolomics in phytomedicine can be arranged into two major classes. These are the ones to understand the molecular effect of medicinal plants and their active components on various diseases, <sup>11,12</sup> health conditions like obesity, <sup>13</sup> and cancer. <sup>14</sup> The others can be defined as the ones to understand the differences between plants obtained from different regions and plant extracts prepared using different conditions. <sup>15,18</sup> Some of these studies include the simultaneous evaluation of the morphological and chemical compositions. <sup>19</sup>

Carthamus tinctorius L., known as Kafesheh (Persian), Aspir (Turkish), and safflower (English) is vastly utilized in Traditional Medicine for various medical conditions, namely dysmenorrhea, amenorrhea, postpartum abdominal pain and mass, trauma, and pain of joints.<sup>20,21</sup> Flavonoids and alkaloids, especially the quinochalcone c-glycoside hydroxysafflor yellow A, *N*-(p-coumaroyl) serotonin, and *N*-feruloylserotonin, are responsible for most of the pharmacological activities of *C. tinctorius*.<sup>22</sup> The seed oil

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of C. tinctorius includes antioxidative flavonoids<sup>23</sup> showing neuroprotective activities.<sup>24</sup> The oil is high in linoleic acid, an unsaturated fatty acid that aids in lowering the blood cholesterol level.<sup>25</sup> In literature, the variation of the fatty acid composition of the seed of C. tinctorius was reported.<sup>26</sup> Besides the seed, the flowers of the C. tinctorius are used in traditional herbal medicine in China, Korea, Japan, and other Asian countries, for treating various ailments such as gynecological, cardiovascular, and cerebrovascular diseases as well as blood stasis and osteoporosis. 22,27 Variation in phenolic composition and antioxidant activity during flower development of *C. tinctorius* was reported.<sup>28</sup> Employment of the different solvent systems and techniques to extract the active metabolites of *C. tinctorius* was performed on several applications.<sup>29-31</sup> The well-known medicinal properties and variations of the chemical components of seed and flower for this unique plant encouraged us to apply recent LC-MS based untargeted metabolomics approaches and data processing strategies to evaluate the variations of the compositions for different extracts of C. tinctorius seed and flowers.

In this study, a quadropole time of flight mass spectrometry - liquid chromatography (Q-TOF LC/MS) based untargeted metabolomic study was designed and applied on 70% ethanol, hexane, and chloroform extracts of both seeds and flowers. A reverse phase chromatographic condition in a gradient elution program was performed for the separation of untargeted components. The raw data were processed, and the total number of the peaks found was compared within each other. Normally, big data from high-throughput metabolomic experiments are commonly visualized using a principal component analysis (PCA) two-dimensional scores plot.<sup>32</sup> Therefore, PCA graphs for the extracts of *C. tinctorius* seeds and flowers were visualized to present the differences. This simple methodology can be used to visualize the metabolite profiles on various phytoextracts of C. tinctorius to evaluate the variations in biological activities.

# **Experimental**

#### Sample collection

Yenice is the first registered spineless safflower variety in Turkey. Its flower colour is deep red, seed color is white. The weight of 1000 seeds ranges from 38 to 40 g. The seed oil content of this variety is 26-28% and protein content is 13%. The Yenice safflower variety was grown in field condition, its petal was collected in July and seed was harvested in August.

# Preparation of the seed and flower extracts of *Carthamus tinctorius* L.

The whole seeds and flowers of the plant material were powdered separately. Three different extracts were prepared using 70% ethanol, hexane, and chloroform while

shaking at 37°C for 24 h. Until the analysis time, the extracts were stored at -20°C. The resulting extracts were concentrated under a vacuum with a rotary evaporator (37-38°C). The form of 70% ethanol was lyophilized. Until the analysis time, extracts were stored at -20°C.

#### Preparation of the samples to be injected

The extracts of both seeds and flowers prepared using 500  $\mu$ L each of 70% ethanol, hexane, and chloroform were dissolved in 1000  $\mu$ L of ethanol:isopropyl alcohol 50:50 (v/v) mixture and vortexed for approximately 1 min. The final volume was filled up to 2000  $\mu$ L with ethanol:isopropyl alcohol 50:50 (v/v) mixture. These samples were diluted with a solvent system [acetonitrile:water 50:50 (v/v) mixture] in an order given as follows: [solvent system: sample 4:4, 4:4, 4:4, 3:3, 2:4, 1:4 (v/v)] as described in the literature.<sup>34</sup>

#### **Chromatographic conditions**

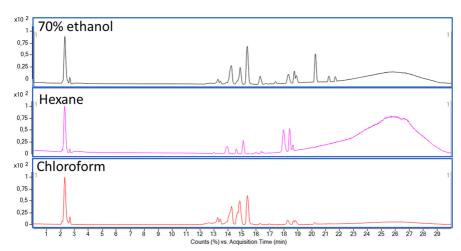
Sample set prepared as given above was injected into an Agilent 6530 LC/MS Q-TOF instrument (Agilent Technologies, 184 Santa Clara, CA) equipped with a C18 column (Agilent Zorbax 1.8 μM, 100 × 2.1 mm). A gradient elution program was performed where the organic and aqueous phases contain 0.1% formic acid. The flow rate was 0.20 mL min<sup>-1</sup> and started with 90% water until the 1st minute, the acetonitrile ratio was elevated linearly to 90% acetonitrile until the 15th minute. The chromatographic conditions were later turned back to starting conditions linearly till the 20th minute and 5 minutes post run was applied for the remaining injections. The scan range for MS devices was 100-1700 m/z. All samples were injected into the system as two replicates in random order. The column temperature was 35°C. The capillary voltage was 4000 V. The mass spectrometer equipped with an ESI probe was operated in positive (ESI+) ion mode.

# **Data processing**

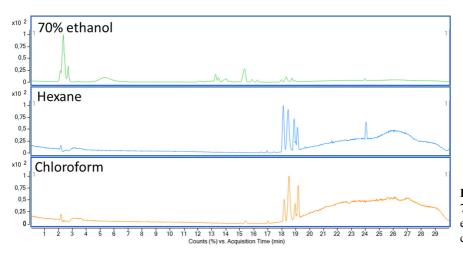
The raw chromatograms for all groups were subjected to data processing through XCMS.<sup>35</sup> Parameters used on XCMS were optimized using isotopologue parameter optimization (IPO).<sup>36</sup> A normalization procedure (using total peak areas to normalize all of the individual peak areas) was performed before the statistical comparison of the groups.<sup>37</sup> False-positive peaks were eliminated using recent data processing strategies.<sup>34</sup> The final number of the peaks found was compared for the extracts and PCA graphs were prepared to compare the results.

#### Results

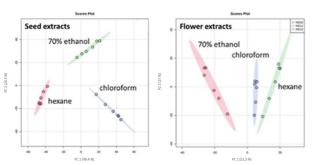
The base peak chromatograms for 70% ethanol, hexane, and chloroform extracts of the seeds (Figure 1) and the flowers (Figure 2) are given below. XCMS is an R



**Figure 1.** Base peak chromatograms for 70% ethanol, hexane, and chloroform extracts of safflower seeds under our experimental conditions.

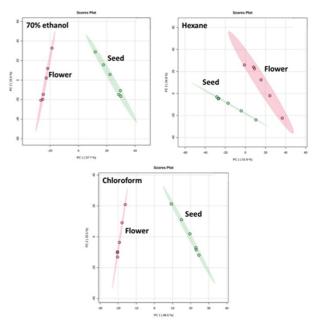


**Figure 2.** Base peak chromatograms for 70% ethanol, hexane, and chloroform extracts of flowers under our experimental conditions.

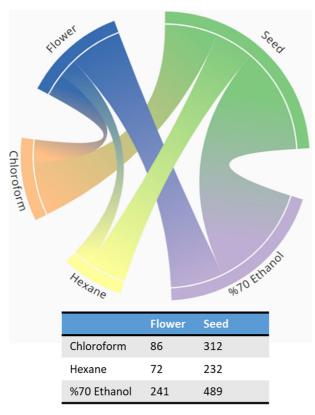


**Figure 3.** PCA graphs demonstrate the differences in the metabolite profiles of seed and flower extracts.

programming language-based platform to process mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. The raw chromatograms for the extracts were processed and a total of 444 peaks were found to be identical on all samples. When the extracts of the seeds and flowers were compared individually for 70% ethanol, hexane, and chloroform, the number of the common peaks for seeds and flowers was



**Figure 4.** PCA graphs demonstrate the effect of each solvent system on the metabolite profiles of seed and flower extracts.



**Figure 5.** Identical peak count comparison from seed and flower on different extraction solvents.

1075, 878, and 623, respectively. The PCA graphs shown in Figure 3 demonstrate the differences in the metabolite profiles for the seeds and flowers. Figure 4 presents the differences in the metabolite profiles for the solvent systems. Figure 5 presents a peak count comparison for only in one extraction method but not in the others from seed and flower on different solvents.

### **Discussion**

As is seen from chromatograms (Figure 1 and 2) and PCA graphs in Figure 3, the metabolite profiles of the extracts for both seeds and flowers were different. Figure 4 confirmed the statistical differences of the metabolites found in 70% ethanol, hexane, and chloroform extracts. The number of the metabolite peaks found in 70% ethanol was the highest in comparison to the hexane and chloroform extracts (Figure 5). There were 1075 common peaks for seeds and flowers detected in 70% ethanol in our experimental conditions. The number of the peaks detected for seeds was higher than the ones detected for flowers for all these solvent systems. Even though the number of metabolites for hexane and chloroform extracts (878 and 623) were different, the concentrations of these metabolites were close to each other for the

flower extracts but not for the seed extracts (Figure 4). The total number of the identical peaks for all samples including different solvent systems for the seeds and flowers was only 444. This was evidence that the composition of the prepared extracts were relatively different. Indeed, the chromatograms (Figure 1 and 2) also verified the results visually.

#### **Conclusions**

Briefly, the untargeted metabolite profiles of *Carthamus* tinctorius L. extracts were different as they were visualized with PCA graphs. The brief methodology presented in this study, which processes Q-TOF LC/MS results with recent data processing strategies used on clinical metabolomics, encouraged us to propose using PCA graphs to evaluate the results of bioactivity tests. As can be seen from the results, different extraction techniques lead to the detection of different groups of compounds. For the purpose of the study, different extraction solvents can be preferred for more polar or more nonpolar compounds. If the proposed methodology in the present study is performed on further applications, the wellknown standardized extracts for lots of plants can be compared with the new ones obtained from the plants in different regions, the ones collected through different protocols, or stored in different conditions. In addition, different solvent systems and/ or processing-cooking strategies could be evaluated to predict biological activity before applying inconvenient potency or bioactivity tests for various samples.

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Author Contributions: Conceptualization, YBK and M $\zeta$ .; methodology OK, NS and AK; software, OK; data curation, OK; writing—original draft preparation, YBK and M $\zeta$ .

<sup>†</sup>Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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